The principles of laboratory animal care as promulgated by the National Society for Medical Research have been observed.

BLOOD COAGULATION FACTOR ACTIVITY IN ENDOTOXEMIA

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In humans with septicemia and in experimental animals that have been given endotoxin, abnormalities of blood coagulation have been observed. Rapaport et al reported a case of Pseudomonas sepsis in a human associated with an antimortem hemorrhagic diathesis, multiple clotting factor deficiencies, and intravascular fibrin thrombi demonstrated post mortem. A similar syndrome associated with pneumococcal sepsis had been reported by Ratnoff and Nebehay (9,24,25).

The purpose of this paper is to describe quantitative alterations in coagulation factor activity in dogs subjected to endotoxemia.

MATERIALS AND METHODS

The principles of laboratory animal care as promulgated by the National Society for Medical Research were observed. Mongrel dogs weighing 9 to 20 kilograms were used in the study. After being anesthetized by pentobarbital sodium given intravenously, the animals were given one injection of endotoxin intravenously. The dose of endotoxin used was calculated to be lethal in 80% of animals. The observed lethality was 67%. This single dose of endotoxin does not produce the generalized Shwartzman reaction in the dog and therefore simulates the majority of human cases of sepsis. Endotoxin was prepared by the method of MacLean and Weil from E. coli (17).

From previous studies in this laboratory of dogs treated in this manner, coagulation abnormalities, manifested by abnormalities in the screening tests described in the following section, develop in approximately 24% of the animals five minutes after injection of endotoxin and 86% of animals four hours after injection of endotoxin.

Collection of Blood: Blood was collected through a polyethylene catheter placed in a femoral artery. For each sample, a fresh catheter was used. All catheters were inserted through the same cut-down site in the artery and passed into the aorta. Each catheter was passed further up the aortic lumen than the previous catheter so as to prevent sampling blood which might be passing over an area of endothelium injured by the previous catheter. Between collection of samples, the artery was clamped with a Pott's Clamp.

Blood used for study of coagulation factor activity was collected from the catheter into a siliconized, graduated centrifuge tube. Nine volumes of whole blood were anticoagulated with one volume of 3.2% sodium citrate. Formed elements and plasma were separated by centrifugation at 2000 g. for 30 minutes. Using siliconized pipets, the plasma was transferred to siliconized test tubes and stored at -20° C.

For analytical chemical examinations, whole blood was collected in plain glass tubes containing 0.1 ml. of 10% sodium EDTA per 10 ml. of whole blood. Intervals of blood collection were: 1. Just prior to injection of endotoxin (control sample); 2. Five minutes after injection of endotoxin; 3. Four hours after injection of endotoxin.

LABORATORY EXAMINATIONS

Hematocrits were done by the micro method, and platelets were counted using a phase contrast microscope and appropriate counting chamber (29). Quantitation of fibrinogen: Fibrinogen was precipitated as fibrin by adding thrombin to an incubation mixture of phosphate buffered saline, pH 6.4, containing calcium and plasma. The fibrin was then quantitated spectrophotometrically using phenol reagent (11,28).

Coagulation Screening Tests:

1. Whole blood clotting times were done in both plain glass and silicone coated tubes by the Lee-White method (29).

2. One-stage Prothrombin Time: Reagents and duplicate samples of test plasma and control plasma were brought to 37° C. in a water bath; then 0.2 ml. of a 1:1 mixture of 0.025 M $CaCl_2$ and thromboplastin was blown from a pipet into a 10 x 75 mm. plain glass tube containing 0.1 ml. of plasma, and a stop watch was simultaneously started. The tube was tilted in air until a clot formed. The stop watch was stopped simultaneously with clot formation (5).

3. Partial Thromboplastin Time: Reagents, and duplicate samples of control plasma and test plasma were brought to 37° C in a water bath. To 0.1 ml. of test and control plasma in separate 10 x

75 mm. test tubes was added 0.1 ml. of a 1:1 mixture of a partial thromboplastin and 2% kaolin. The mixture of plasma, kaolin, and partial thromboplastin was incubated for two minutes with intermittent gentle agitation. At the end of this two minute incubation, 0.1 ml. of 0.025 M $CaCl_2$ was blown into the mixture and a stop watch started simultaneously. The tube was tilted in air, and the interval required for clot formation was timed (5,16,19,22,26).

4. Specific Quantitative Assays for Clotting Factor Activity: A quantitative estimate of the activity of a given clotting factor in a plasma can be made by testing the ability of this plasma to correct the clotting defect in a plasma known to be deficient in the factor to be quantitated. Technical details for performing assays are given in references cited (1,5,8,13,28). Plasmas from well studied humans known to be deficient in only one of the following factors, VII, IX, X, XII and plasma from a dog known to be deficient only in factor VIII were supplied by Dr. Kenneth M. Brinkhous and Dr. George Penick, Department of Pathology, University of North Carolina. Factor V deficient plasma was artificially prepared by incubating oxalated dog plasma at 37° C until the one-stage prothrombin time was 20-30 sec. Factor XI deficient plasma was prepared artificially by adsorption of human plasma with "Filter-Cel" (13). Prothrombin was quantitated by a modification of the method of Owren (5).

Normal Values and Control Plasma: To establish normal values for the laboratory examinations to be done during the experiment, blood from 31 normal dogs was studied. Results are shown in Table 1.

Table 1
Normal Values

Test	Mean	Range
Fibrinogen	333 mgm %	175 - 499 mgm %
Total Protein	6.4 gm %	5.5 - 7.3 gm %
P. T.	8.0 sec.	6.6 - 10.2 sec.
P. T. T.	19.0 sec.	14.2 - 25.7 sec.
Platelet count	246,000 mm^3	55,000 - 391,000/mm^3
Whole Blood Clotting Time:		
In Glass	6.5 min.	3 - 10 min.
In Silicone	35 min.	14 - 97 min.
HCT.	49%	37 - 57%

Table 1-A shows the range of activity of the various clotting factors in the normal plasma of the twelve dogs used in this experiment. The activity is expressed as percent of the activity in the plasma of the control dog described in the following paragraph.

Table 1-A
Normal Values for the Activity
of the Dog's Clotting Factors*

Factor	Range of Activity
Prothrombin	84 to > 100%
Factor V	75 to > 100%
Factor VII	55 to > 100%
Factor VIII	70 to > 100%
Factor IX	78 to > 100%
Factor X	56 to > 100%
Factor XI	63 to > 100%
Factor XII	96 to > 100%

*See text for discussion

As a control for the one-stage prothrombin time, partial thromboplastin time, and factor assays, plasma from one normal male dog was used throughout the experiment. The dog had not manifested any bleeding tendencies while under observation in the kennels, and his coagulation screening tests were within normal limits.

RESULTS

Twelve dogs were studied in detail. These animals were purposely selected for detailed study because they developed unequivocal coagulation abnormalities. All twelve animals had abnormalities at four hours, whereas six of the twelve were abnormal at five minutes after injection of endotoxin.

One-Stage Prothombin Time and Partial Thromboplastin Time: The clotting time of plasma as measured by these tests reflects clotting factor activity. The coagulation defect which developed at five minutes manifested itself by a prolongation of the partial thromboplastin time in all six animals and the one-stage prothrombin time in two of the six animals. Four hours after endotoxin, the partial thromboplastin time was prolonged in all twelve animals, and the one-stage prothrombin time was prolonged in eight of the twelve. The mean and range of these tests are shown in Table 2.

Table 2
Prothrombin and Partial Thromboplastin Times*

	P. T.		P. T. T.	
Sample	Mean	Range	Mean	Range
Control	7.2	6.6 - 8.0	19.1	15.8 - 22.7
**5 min.	7.6	6.6 - 8.4	40.0	29.9 - 56.9
4 hr.	9.5	7.3 - 18.6	35.0	23.6 - 66.7

*Expressed in seconds
**Data derived from the six animals that developed abnormalities at this interval after endotoxin.

The whole blood clotting time in both plain glass and siliconized tubes was within normal limits on the control sample of blood from all dogs. Five minutes after endotoxin, both the plain glass and silicone clotting times were prolonged. At four hours after endotoxin, all clotting times done in plain glass tubes were normal, but the silicone clotting time was prolonged in eleven of the twelve animals.

Platelets: A marked drop in platelet count had occurred by five minutes after injection of endotoxin. Four hours after endotoxin, the platelet count of all animals had increased. Table 3 shows the mean and range of platelet counts.

Table 3
Platelet Counts*

Sample	Mean	Range
Control	284,000	180,000 - 487,000
5 min. after endotoxin	16,000	3,000 - 41,000
4 hr. after endotoxin	154,000	85,000 - 247,000

*Expressed as thousands per mm^3.

Fibrinogen: The concentration of fibrinogen was essentially unchanged five minutes after endotoxin injection. A slight decrease had occurred at four hours; however, the reduction was not sufficient to cause abnormalities of coagulation. Table 4 shows the mean and range of fibrinogen concentration.

Table 4
Fibrinogen Concentration*

Sample	Mean	Range
Control	359	182 - 676
5 min. after endotoxin	306	190 - 445
4 hr. after endotoxin	266	170 - 376

*Expressed as milligrams percent.

Prothrombin and Factors V, VII, VIII, IX, X, XI, XII: Table 5 shows the means of the decrease in activity of these clotting factors. When the data from the twelve animals are analyzed collectively, as presented in Table 5, a slight to moderate decrease in activity of all factors is present both five minutes and four hours after the injection of endotoxin. If the data from each animal are considered individually, the following additional conclusions can be drawn:

1. When an animal developed a coagulation defect, the activity of several, but not necessarily all factors is decreased.

2. The degree of activity lost by any one of the factors studied varies from animal to animal.

Table 5
Loss of Clotting Factor Activity*

	Interval After Endotoxin			
	5 min.		4 hr.	
Factor	Mean	Range	Mean	Range
Prothrombin	25	2 - 50	38	9 - 53
Factor V	28	10 - 40	64	45 - 90
Factor VII	21	4 - 39	66	13 - 90
Factor VIII	47	20 - 90	32	0 - 90
Factor IX	45	0 - 84	42	5 - 87
Factor X	15	0 - 44	60	25 - 90
Factor XI	68	37 - 90	54	0 - 86
Factor XII	37	0 - 89	34	0 - 53

*Expressed as percent of the total activity originally present in the animals' plasma before endotoxin was given.

DISCUSSION

The coagulation abnormality associated with endotoxemia results from slight to moderate decrease in the activity of several coagulation factors. Thrombocytopenia, which is severe immediately after endotoxin is given, may contribute to the overall clotting defect at that time, but four hours after endotoxin is given, the platelets are present in sufficient numbers to maintain their role in hemostasis. Although a slight decrease in fibrinogen did occur, sufficient fibrinogen was present in the plasma of all animals studied at both five minutes and four hours after injection of endotoxin for normal clot formation. The mechanism by which the activity of the coagulation factors was reduced cannot be specifically defined from this study. Mechanisms to be considered which might collectively or individually cause a reduction in factor activity are:

1. Hemodilution
2. Increased fibrinolytic activity in the plasma
3. Circulating anticoagulants
4. Utilization in the formation of fibrin.

In this experiment, hemodilution can be excluded. During the four hour interval of the experiment, the only fluids injected into the animals were the small quantities used as vehicles for the anesthetic and endotoxin. This amounted to less than 20 cc per dog. As an indicator of changes in plasma volume, the total protein concentration and hematocrit were determined. (Table 6). These data demonstrate no hemodilution.

Table 6
Total Protein* and Hematocrit**

Sample	Total Protein Concentration		Hematocrit	
	Mean	Range	Mean	Range
Control	6.7	6.0 - 8.4	51	45 - 60
5 min after endotoxin	6.0	5.4 - 6.4	55	47 - 68
4 hr after endotoxin	5.7	4.8 - 6.0	56	45 - 68

*Expressed in grams percent
**Expressed in percent

The total protein concentration dropped slightly and the hematocrit increased slightly over the four hour period of the experiment. These findings most likely are the result of loss of protein-rich fluid from damaged capillaries in the gastrointestinal tract and liver of the dog. Autopsy studies made by one of the authors (R.L.W.) on dogs receiving a comparable dose of endotoxin revealed severe capillary congestion in the gut and sinusoidal congestion in the liver.

Circulating Anticoagulants: The following observations by other investigators make it necessary to consider circulating anticoagulants as a possible mechanism by which factor activity is reduced. In the human case of septicemia reported by Ratnoff (25), results of screening tests suggested the presence of a circulating anticoagulant. In experimental animals, Gans et al have demonstrated a slight rise in antithrombic activity in the dog during the first five minutes after injection of endotoxin (6). In an extensive review of the subject, Margolis et al pointed out that these substances may either block a specific coagulation factor or may block one of three phases of coagulation (18).

Fibrinolytic Activity: In addition to digesting fibrin and fibrinogen, it has been demonstrated in vitro that plasmin can digest factors V, VIII, and IX rapidly and, if incubated long enough, prothrombin (4). Further, it has been shown that break-down products formed when plasmin digests fibrin have a heparin-like action in that they inhibit the formation of fibrin from fibrinogen (15). Because of these observations, an increase in plasmin activity would have to be considered as a possible mechanism by which clotting factor activity might be reduced.

Gans and Krivit have assayed plasmin in the plasma of dogs after administration of endotoxin. They found no increase in circulating plasmin, but did demonstrate a decrease in plasminogen. Their interpretation of this observation was that plasmin was adsorbed onto fibrin which was forming in the animal (7).

Utilization in Formation of Fibrin: Intravascular coagulation has been studied in the experimental animal by injection of both thrombin and thromboplastin. In both experimental models, thrombocytopenia, a decrease in fibrinogen concentration and a decrease in activity of other coagulation factors occurred. Of the factors studied, only factor VII and factor IX had no decrease in activity (10, 20,21, 23). These changes are similar to those observed during endotoxemia except for the decrease in factors VII and IX which occurred during endotoxemia. If intravascular fibrin formation is associated with endotoxemia, endotoxin must in some way initiate the clotting process. When injected intravenously, endotoxin is largely cleared from the blood. However, of that amount remaining in the blood, 97% is localized in platelets (12). Des Prez et al have demonstrated that when exposed to endotoxin, platelets agglutinate and release platelet factor III, which in conjunction with other plasma clotting factors, contributes to the formation of plasma thromboplastin. Rodrequez-Erdmann has shown that endotoxin does not activate prothrombin but does activate Hageman factor (factor XII) in vitro (27). These data suggest the mechanism by which endotoxin may initiate coagulation is by initiating plasma thromboplastin formation through its action on platelets and factor XII.

The data from this experiment demonstrates only that a decrease in activity of coagulation factors occurs but does not allow definition of the mechanisms by which the activity is reduced.

More detailed studies of the fibrinolytic system, endogenous anticoagulants, and intravascular fibrin formation will be required to more specifically define their role in reduction of clotting factor activity.

SUMMARY

Twelve dogs were subjected to endotoxemia and the activity of coagulation factors was studied quantitatively. Coagulation studies were performed on the dogs' normal plasma and on plasmas collected 5 minutes and 4 hours after endotoxin administration. All dogs had coagulation abnormalities 4 hours after endotoxin, and six of the twelve had abnormalities 5 minutes after endotoxin. With the exception of the platelet counts, the coagulation abnormality at 5 minutes and 4 hours was similar. It was characterized by a prolongation of the one-stage prothrombin time and/or partial thromboplastin time, a slight reduction in fibrinogen concentration, and a variable degree of deficiency in the activity of factors II, V, VII, VIII, IX, X, XI and XII.

This study does not define the mechanisms by which the activity of these factors are reduced; however, the several possible mechanisms are discussed.

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